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14. ABSTRACT Current prevention focuses on oral administration of chemopreventive agents which decreases breast cancer incidence but increases the risk for secondary treatment-induced disease and may not be effective in preventing those lesions that are estrogen receptor (ER) negative. We hypothesize that programmed cell death is dysregulated in premalignant breast cells which permits these cells to avoid cell death. Our studies indicate the ductal carcinoma in situ cell line DCIS3A overexpresses the anti-apoptotic proteins Bcl-2 and Bcl-xL compared to normal breast tissue. In addition, we have shown that DCIS3a treated with a bispecific antisense oligonucleotide against bcl-2 and bcl-xl down regulates expression of both proteins. These studies also show an increase in programmed cell death in the DCIS3A cell line after treatment with the bispecific antisense oligo bcl-2/bcl-xl alone but not with tamoxifen alone nor a synergic effect in combination with the antisense oligo. Finally, cells obtained from breast ductal lavage appear to express BCL-2, BCL-XL, and BAX which suggests these cells may be already avoiding programmed cell death.					
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TITLE:

Breast Cancer Prevention by Inducing Apoptosis in DCIS Using Breast Ductal Lavage

INTRODUCTION:

Although great strides have been made in breast cancer screening and treatment, it remains the second highest cause of cancer-related deaths for women in the United States. Current prevention has focused on oral administration of chemopreventive agents which appears to decrease breast cancer incidence but increases the risk for secondary treatment-induced disease. In addition, these chemopreventive agents may not be effective in preventing those lesions that are estrogen receptor (ER) negative based on its primary function of suppressing cell proliferation by blocking the estrogen receptor. We hypothesize that programmed cell death (PCD) is dysregulated in premalignant and malignant breast cells which permits both ER-positive and ER-negative cells to avoid cell death. We intend to investigate whether treating premalignant breast cells with a molecular genetic-based agent (antisense bcl-2/bcl-xL oligonucleotide) may be effective alone or in concert with tamoxifen treatment to induce cell death in both ER-positive and ER-negative cells. Ultimately, we envision using the newly developed technique of breast ductal lavage to not only screen women for increased risk in developing breast cancer, which is currently being performed, but to also use this technique to delivery genetic-based preventive agents and/or a chemopreventive agent directly to the breast ductal lobe of these high risk individuals thus eliminating any potential for secondary treatment-induced diseases.

BODY:**Statement of Work**

Task 1. Determine expression pattern of the PCD regulatory genes bcl-2, bax, and bcl-xL in primary DCIS cultures (Months 1-6):

- a. Protein analysis of Bcl-2, Bax, and Bcl-xL using Western blotting and immunofluorescent staining.

This task has been accomplished once **DoD gave approval on June 6, 2006** to work with the proposed five DCIS and two normal primary explant breast cell lines provided by Jean Latimer, PhD from the University of Pittsburgh Medical Center. Dr. Latimer sent two DCIS cell lines (designated DCIS3A and DCIS4) and two normal breast cell lines (designated BRL23 and BRL24). Unfortunately the only cell lines that could be cultured to sufficient cell numbers to accomplish this task were DCIS3A and BRL23. DCIS4 and BRL24 never grew sufficiently to be analyzed. However, we did perform Western blot analysis to determine the endogenous protein levels for Bcl-2, Bcl-xL, and Bax for both DCIS3A and BRL23. As can be seen in Figure 1, DCIS3A expresses both Bcl-2 and Bcl-xL but the normal breast cell line BRL23 does not express any detectable amounts of either protein.

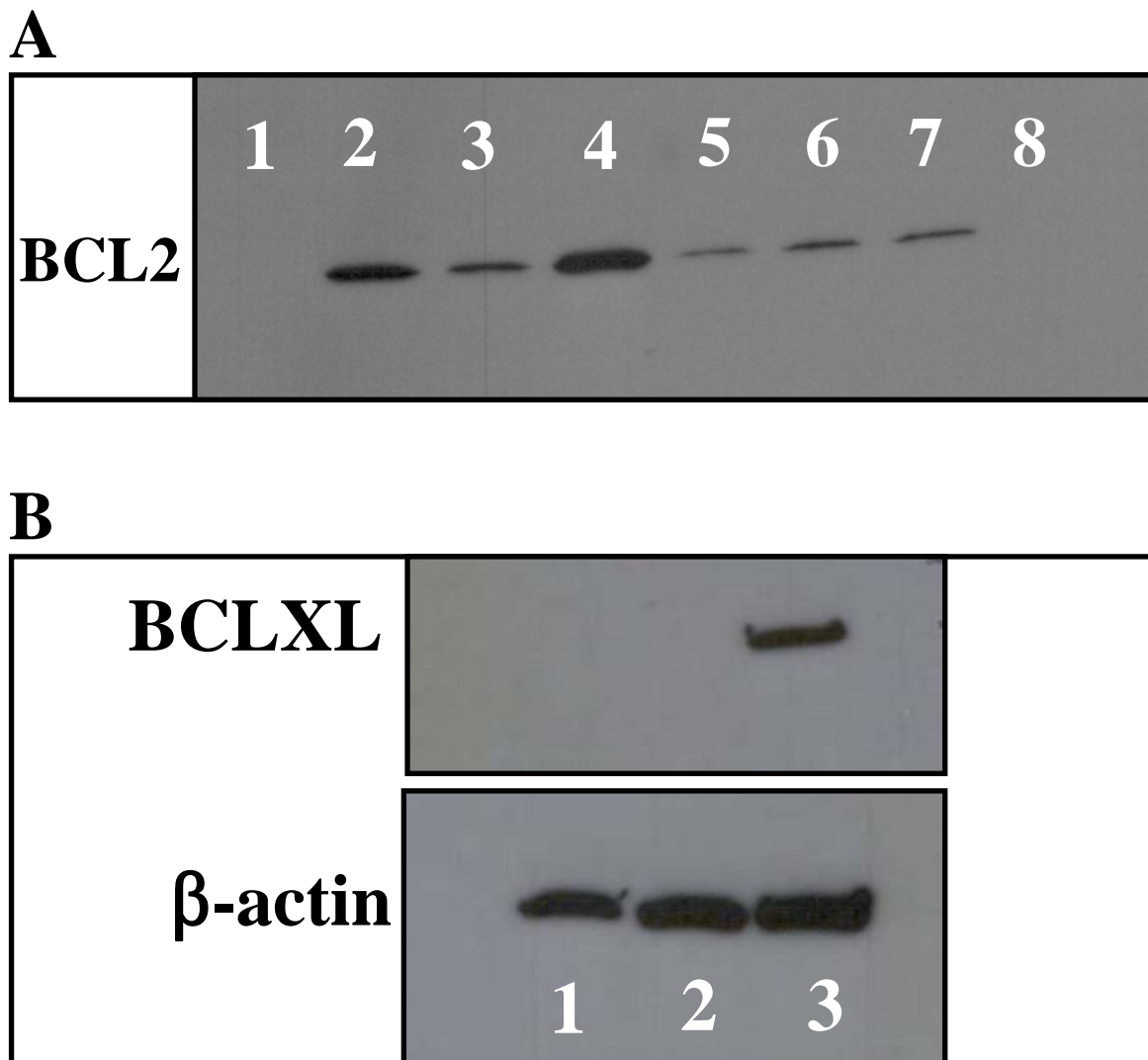


Figure 1. Western blot analysis of endogenous levels of Bcl-2 and Bcl-xL. Equal amounts of total protein (50 μ g), as measured by spectroscopy using a standard protocol, was loaded into each lane of a 4-20% gradient polyacrylamide gel. Following protein transfer to a PVDF membrane, the blots were incubated in primary antibody (Bcl2 1:500, Dako; Bcl-xL 1:1000, Zymed; or β -actin 1:2000, Chemicon) overnight at 4° followed by incubation with a HRP secondary antibody (1:1000, Dako) for 2 hrs at room temperature. Chemiluminescence staining (Pierce) was performed followed by exposure to autoradiograph film. **A.** lanes 1 and 8: normal breast primary explant cell line BRL23; lanes 2-7: DCIS3 primary explant cell line **B.** lanes 1 and 2: normal breast primary explant cell line BRL23; lanes 3: DCIS3 primary explant cell line.

This observation supports our hypothesis that programmed cell death is dysregulated in DCIS as compared to normal breast tissue. This gave us the evidence to perform *Task 2*.

Task 2. Determine whether down-regulation by genetic manipulation of the anti-apoptotic genes bcl-2 and/or bcl-xL alone or in conjunction with physiological

preventive doses of tamoxifen has the highest induction of PCD in primary DCIS cell cultures (Months 6-18):

- Treatment with antisense and control oligonucleotides and/or tamoxifen.
- Protein analysis of Bcl-2, Bax, and Bcl-xL using Western blotting and immunofluorescent staining.
- Quantify mRNA for bcl-2 or bcl-xL using a PCR-based assay.
- Determine effect of treatment on programmed cell death markers using assays for DNA fragmentation and caspase activation.

We have also completed *Task 2* using the DCIS3A cell line. Our studies, based on the data obtained in *Task 1*, involved treating the DCIS3A cells with a bi-specific antisense bcl-2/bcl-xl oligonucleotide and/or with 4-hydroxytamoxifen (Sigma). We first determined by Western blot if liposome-based transient transfection with the bi-specific antisense oligonucleotide for 24 hr down regulated either BCL-2 or BCL-XL or both. As can be seen in Figure 2, treatment with the antisense bcl-2/bcl-xl oligonucleotide clearly down regulated both BCL-2 (decreased by 6.25-fold) and BCL-XL (decreased by 5.6-fold) as compared to medium and the non-sense oligonucleotide control.

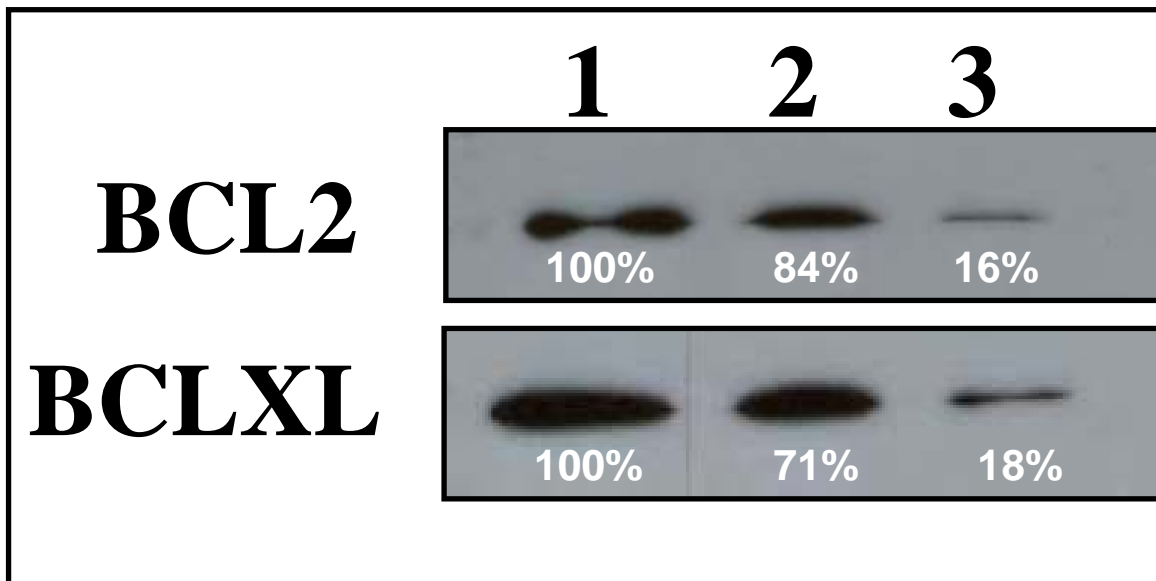


Figure 2. Western blot analysis of levels of BCL-2 and BCL-XL after transfection of the DCIS3A cell line with a bi-specific antisense bcl-2/bcl-xl oligonucleotide for 24hr. DCIS3A cells were liposomally transiently transfected with 0.5µg nonsense control or 0.5µg antisense bcl-2/bcl-xl oligonucleotides for 24 hrs. Equal amounts of total protein (50µg), as measured by spectroscopy using a standard protocol, was loaded into each lane of a 4-20% gradient polyacrylamide gel. Following protein transfer to a PVDF membrane, the blots were incubated in primary antibody (Bcl2 1:500, Dako; Bcl-xL 1:1000, Zymed) overnight at 4° followed by incubation with a HRP secondary antibody (1:1000, Dako) for 2 hrs at room temperature. Chemiluminescence staining (Pierce) was performed followed by exposure to autoradiograph film. Autoradiographs were quantified using ImageJ software. **Lane 1:** Medium control, 100% BCL-2 and 100% BCL-XL; **Lane 2:** Non-sense control, 84% BCL-2 and 71% BCL-XL as compared to medium control; **Lane 3:** Antisense bcl-2/bcl-xl oligonucleotide, 16% BCL-2 and 18% BCL-XL as compared to medium control.

We next determined whether the down regulation of BCL-2 and BCL-XL after liposome-based transient transfection with the bi-specific antisense bcl-2/bcl-xl oligonucleotide for 24 hr was enhanced when treated in conjunction with 5 μ M tamoxifen (physiological dose given in chemoprevention trials). As seen in Figure 3, we observed a decrease in BCL-2 and BCL-XL, regardless of treatment. However, the greatest decrease in expression of both proteins (7.7-fold for BCL-2 and 4-fold for BCL-XL as compared to medium control) occurred when treated with the bi-specific antisense bcl-2/bcl-xl oligonucleotide alone. It does not appear that the combination of the antisense oligonucleotide with tamoxifen has any synergistic effect on the level of expression of either protein.

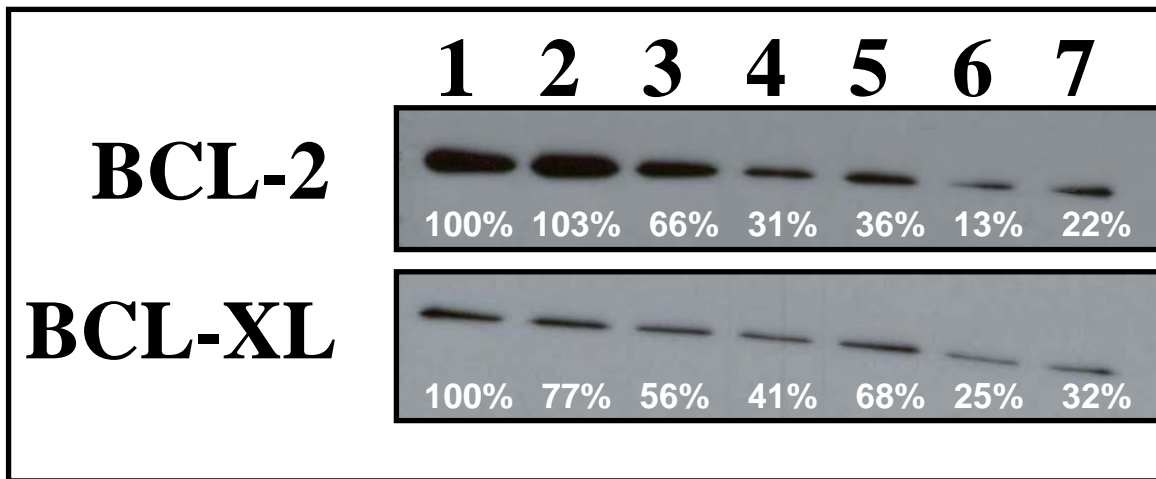


Figure 3. Western blot analysis of levels of BCL-2 and BCL-XL after transfection of the DCIS3A cell line with a bi-specific antisense bcl-2/bcl-xl oligonucleotide alone or in combination with tamoxifen for 24hr. DCIS3A cells were liposomally transiently transfected with 0.5 μ g nonsense control or 0.5 μ g antisense bcl-2/bcl-xl oligonucleotides alone or in combination with 5 μ M tamoxifen for 24 hrs. Equal amounts of total protein (50 μ g), as measured by spectroscopy using a standard protocol, was loaded into each lane of a 4-20% gradient polyacrylamide gel. Following protein transfer to a PVDF membrane, the blots were incubated in primary antibody (Bcl2 1:500, Dako; Bcl-xL 1:1000, Zymed) overnight at 4° followed by incubation with a HRP secondary antibody (1:1000, Dako) for 2 hrs at room temperature. Chemiluminescence staining (Pierce) was performed followed by exposure to autoradiograph film. Autoradiographs were quantified using ImageJ software. **Lane 1:** Medium control, 100% BCL-2 and 100% BCL-XL; **Lane 2:** Ethanol carrier control, 103% BCL-2 and 77% BCL-XL; **Lane 3:** Tamoxifen, 66% BCL-2 and 56% BCL-XL; **Lane 4:** Non-sense control, 31% BCL-2 and 41% BCL-XL; **Lane 5:** Nonsense and tamoxifen, 36% BCL-2 and 68% BCL-XL; **Lane 6:** Antisense bcl-2/bcl-xl oligonucleotide, 13% BCL-2 and 25% BCL-XL; **Lane 7:** Antisense and tamoxifen, 22% BCL-2 and 32% BCL-XL.

Finally for *Task 2*, we determined whether the bi-specific antisense bcl-2/bcl-xl oligonucleotide alone or in conjunction with physiological preventive doses of tamoxifen had the highest induction of PCD in the DCIS3A cells. We determined the effect of this treatment on the programmed cell death pathway by performing a pan caspase activation

assay (Immunocytochemistry Technologies) using a fluorescent microscopy protocol which we determined both caspase activation and the presence of apoptotic bodies (Figure 4).

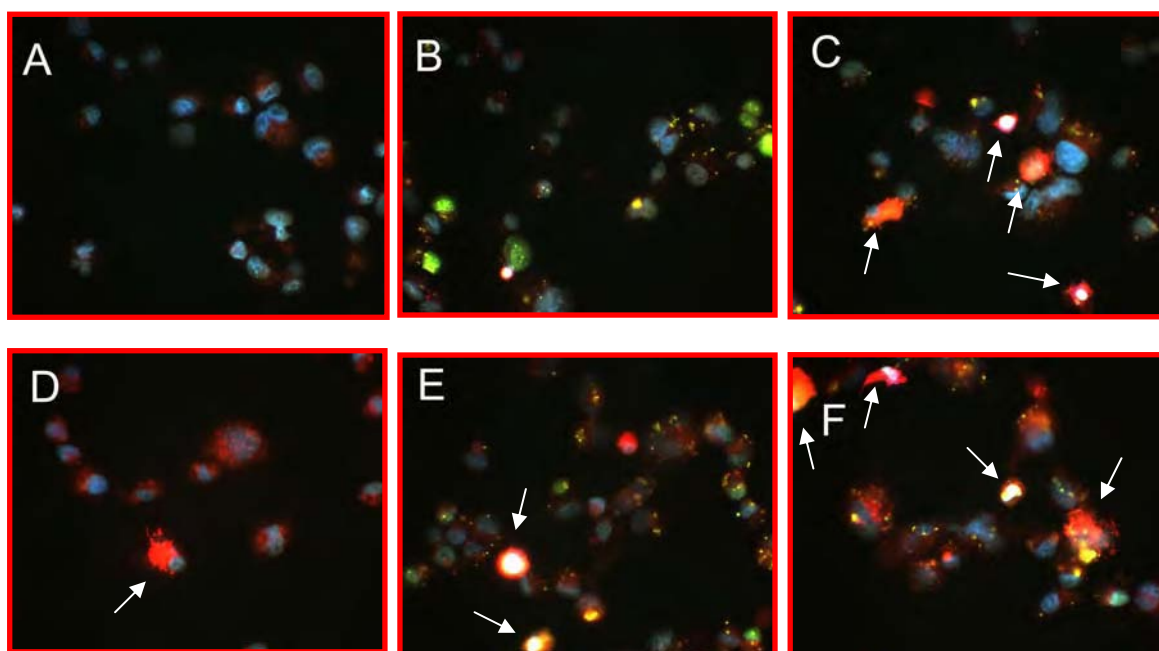


Figure 4. Induction of programmed cell death in the DCIS3A primary explant cell line after treatment with an antisense bcl-2/bcl-xl oligonucleotide and/or 4-hydroxytamoxifen. DCIS3A cells were treated with 0.5µg nonsense control or 0.5µg antisense bcl-2/bcl-xl oligonucleotides and treated with 5µM 4-hydroxytamoxifen as indicated for 24 hrs. Nuclei are stained with Hoechst 33342 (blue), oligonucleotides are labeled with Fam (green), caspase activation identified by VAD cleavage (red) and indicated with arrows. **A.** Medium control **B.** nonsense oligonucleotide **C.** antisense bcl-2/bcl-xl oligonucleotide **D.** 4-hydroxytamoxifen **E.** nonsense oligonucleotide and 4-hydroxytamoxifen **F.** antisense bcl-2/bcl-xl oligonucleotide and 4-hydroxytamoxifen.

The results of this analysis are summarized in Table 1. This analysis indicates that all treatment conditions induce an increase in programmed cell death with the highest induction with treatment of antisense bcl-2/bcl-xl alone or in combination with tamoxifen. Again, there does not appear to be a synergistic effect with the combination of the antisense oligonucleotide and tamoxifen.

Table 1.

Treatment Condition	Caspase Activation and/or Apoptotic Bodies (%)
Medium control	1
Nonsense oligonucleotide control	6
Antisense bcl-2/bclxl oligonucleotide	9
4-hydroxytamoxifen	2
Nonsense oligo & 4-hydroxytamoxifen	7
Antisense oligo & 4-hydroxytamoxifen	10

NA – Not appropriate

Task 3. Determine expression pattern of the PCD regulatory genes bcl-2, bax, and bcl-xL in cells obtained by breast ductal lavage (Months 18-24):

- a. Protein analysis of Bcl-2, Bax, and Bcl-xL using immunofluorescent staining.

After getting **approval from the DoD on March 12, 2006**, breast ductal lavage samples were obtained from twenty-nine informed patients. Unfortunately, the vast majority of these lavage samples did not have any cells to evaluate and only a few samples had a very limited number of cells (at most hundreds) to evaluate and most of those cells were not ductal in origin. Most cells obtained have been squamous epithelium from the nipple, red blood cells, and macrophages and/or histiocytes (Figure 5).

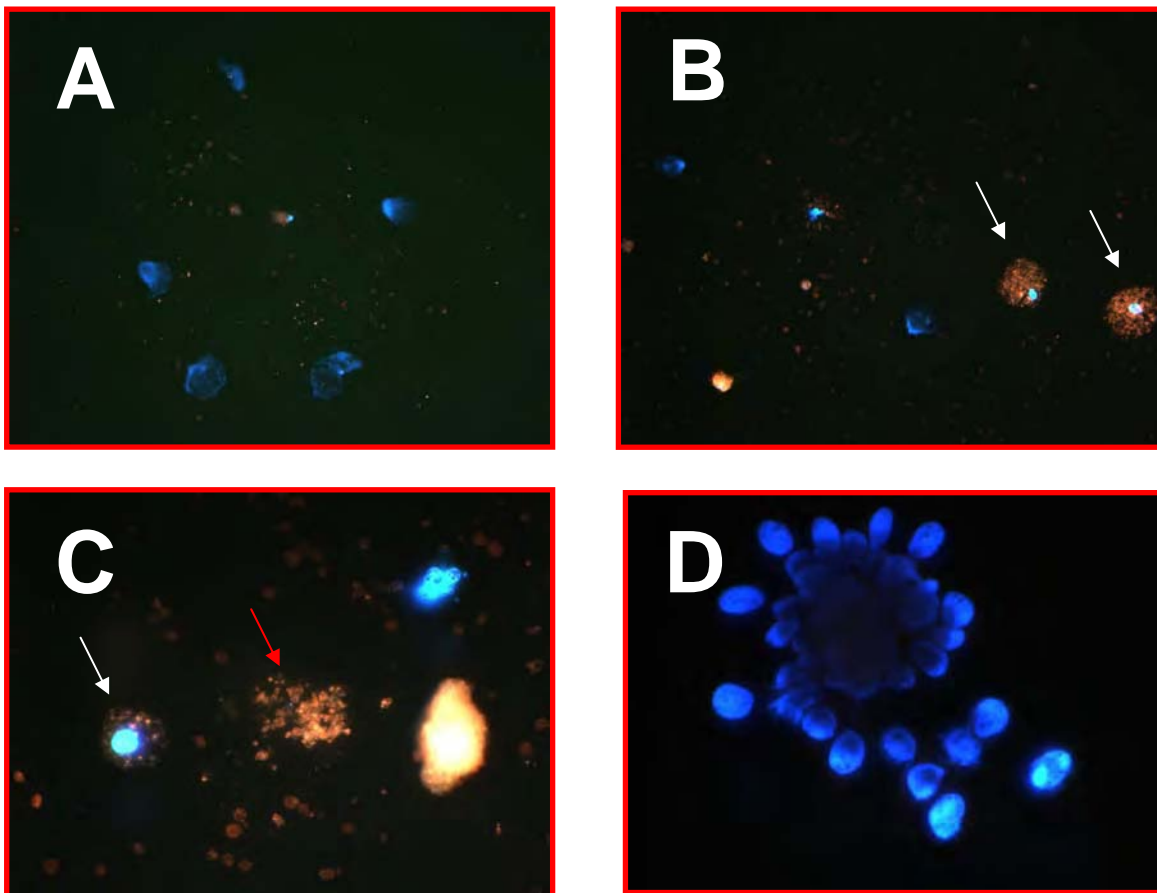


Figure 5. Fluorescence microscopic analysis of cells obtained from ductal lavage procedure. Nuclei were stained with DAPI. **A.** Squamous epithelium from nipple. **B.** Histiocytes (white arrow). **C.** Macrophage (white arrow) and red blood cells (red arrow). **D.** Breast duct epithelial cells.

This lack of success of obtaining sufficient samples is not limited to our institution but has been published by others as well [K. Visvanathan et al. "The Reliability of Nipple Aspirate and Ductal Lavage in Women at Increased Risk for Breast Cancer—a Potential

Tool for Breast Cancer Risk Assessment and Biomarker Evaluation” *Cancer Epidemiol Biomarkers Prev* 2007;16(5):950–55]. For those few samples that had a minimal number of breast duct epithelial cells, we did attempt to accomplish *Task 3*. We determined whether cells obtained from breast ductal lavage expressed BCL-2, BCL-XL, and/or BAX. Figure 6 shows some cells indeed appear to be expressing these proteins. Therefore, these results suggest that cells that are being released into the breast ducts may already be abnormal to the extent of overexpressing PCD proteins and could potentially be targets for genetic manipulation with antisense bcl-2/bcl-xl oligonucleotide.

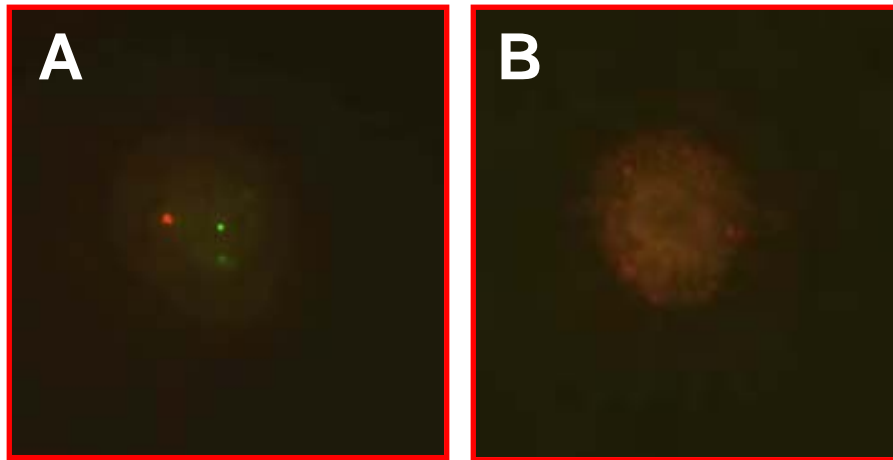


Figure 6. Expression of BCL-2, BCL-XL, and BAX in breast duct epithelial cells obtained from breast ductal lavage. Protein expression was determined by immunofluorescence staining using antibodies specific for Bcl-2 (monoclonal, Santa Cruz), Bax (monoclonal, Santa Cruz), and Bcl-xL (polyclonal, Santa Cruz). Nuclei were stained using Hoechst 33342. Cells were fixed with paraformaldehyde then washed with PBS. Non-specific binding sites were suppressed using 3% BSA in PBS. Antibodies for Bcl-2 (1:100) and Bax (1:100) were detected using Alexa Fluor 568 while Bcl-xL (1:100) was detected using Alexa Fluor 488 fluorochrome-conjugated antibodies (Molecular Probes, 1:00). **Panel A:** A breast duct epithelial cell expressing BCL-2 (Red) and BCL-XL (Green) in the nucleus. **Panel B.** A breast duct epithelial cell expressing BAX (Red) in the cytoplasm.

Task 4. Determine whether down-regulation by genetic manipulation of the anti-apoptotic genes bcl-2 and/or bcl-xL alone or in conjunction with physiological preventive doses of tamoxifen has the highest induction of PCD in cells from breast ductal lavages (Months 24-36).

- a. Treatment with antisense and control oligonucleotides and/or tamoxifen.
- b. Protein analysis of Bcl-2, Bax, and Bcl-xL using Western blotting and immunofluorescent staining.
- c. Quantify mRNA for bcl-2 or bcl-xL using a PCR-based assay.

- d. Determine effect of treatment on programmed cell death markers using assays for DNA fragmentation and caspase activation.

The vast majority of these lavage samples did not have any cells to evaluate and only a few samples had a very limited number of cells (at most hundreds) to evaluate and most of those cells were not ductal in origin. This has made *Task 4* impossible to complete.

KEY RESEARCH ACCOMPLISHMENTS:

- Shown the anti-apoptotic proteins Bcl-2 and Bcl-xL are overexpressed in DCIS3A cells as compared to normal breast tissue.
- Treatment of DCIS3A cells with antisense bcl-2/bcl-xL oligonucleotides down regulates both BCL-2 and BCL-XL.
- Treatment of DCIS3A cells with antisense bcl-2/bcl-xL oligonucleotides and/or 4-hydroxytamoxifen induces programmed cell death.
- Breast duct epithelial cells appear to express BCL-2, BCL-XL, and BAX.

REPORTABLE OUTCOMES: N/A**CONCLUSIONS:**

These studies support our hypothesis by showing that DCIS3A cells have a dysregulated programmed cell death pathway and are overexpressing Bcl-2 and Bcl-xL compared to normal breast tissue. We have also shown with this data that treatment of DCIS3A cells with antisense bcl-2/bcl-xl oligonucleotides and/or 4-hydroxytamoxifen induces programmed cell death. In addition, this study indicates that improvements must be made the breast ductal lavage procedure to obtain sufficient cells for analysis in order to have any potential to be used as a molecular diagnostic screening tool.

REFERENCES:

Abstract entitled "Treatment with an antisense oligonucleotide against bcl-2 and bcl-xl but not tamoxifen induces programmed cell death in a ductal carcinoma in situ primary tissue explant." to be presented at the *AACR Special Conference Advances in Breast Cancer Research; Genetics, Biology, and Clinical Applications*; San Diego CA October 17-20, 2007.

APPENDICES:**LIST OF PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT:**

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